TRANSFORMATION OF PNEUMOCOCCAL TYPES

Introduction

I. Brief historical review

A. Griffith - 1928, unencapsulated, avirulent R-variants of one specific type transformed into fully encapsulated and virulent cells of a different specific type

Example and technique:

Type II R → Type III S

R strain incapable of causing fatal bacteremia

Heated Type III suspension contained no viable organisms. Thus, R cells acquired capsular structure and serological specificity of Type III.

Slide 1 illustrates phenomenon

1. Numerous types of pneumococcus

2. Type specificity - dependent on presence in capsule of a polysaccharide chemically distinct and serologically specific for each type.

3. Capsule is immunological unit of type specificity and accessory structure essential in determining invasive capacity or organism.

4. Under certain cultural conditions enzymes concerned in synthesis of capsular material may be suppressed or lost. With loss of capsule cell loses its type specificity and invasive properties. → R form. Reaction often reversible - animal passage; anti-R serum. Reversion always back to type from which it came. Reversion S ⇌ R different from transformation.

5. In latter instance, R acquired the capsule and type specificity of the encapsulated cells used to induce the reaction.

B. Confirmation of phenomenon

1. Newfeld and Levinthal - 1928 } abroad
2. Baurhenn - 1932 }
3. Dawson - 1930 - in laboratory

C. Induction in vitro

1. Dawson and Sia - 1931 (intact and heated cells)
Grew R cells in media containing anti-R serum and heated S cells.

2. Alloway 1932 - (cell-free extracts; transforming principle in soluble form)
Berkefeld filtered

Hence in test tube as in mice, showed transformation induced and selectively determined by type specificity of S cells used.

D. Virus - fibroma → myxoma

1. Berry and Dedrick - 1936

Living fibroma virus + heat-killed myxoma → rabbits induced myxomatosis.

2. Berry 1937

Induction by suspensions of washed elementary bodies of myxoma.

3. Confirmation by number of investigators.

Present Study - more detailed analysis of phenomenon; attempt to determine chemical nature of transforming principle. Lack

Model: Type II → Type III.

II. Cultural Conditions - certain conditions requisite { Recognition of these factors facilitate obtaining consistent + reproducible

A. Broth - optimal for growth. Individual and unpredictable variations in capacity to support transformation. These largely eliminated by charcoal adsorption (MacLeod and Mirick)

B. Serum or serous fluid

1. Anti-R serum first used because of capacity to promote reversion of R → homologous S.

2. Alloway used ascitic and chest fluid and normal swine serum (contain anti-R)

3. Present study - human pleural and ascitic fluids used.

Effectiveness of different lots varies. Differences not dependent on content of R antibodies, suggesting other factors involved. No criteria

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4. Enzyme - various animal sera irrespective of immune properties contain enzyme that destroys transforming principle.

Enzyme inactivated at 60-65°C. Heating may render ineffective sera effective.

5. Unknown factor - suggested by fact that some sera containing anti-R and enzyme heat inactivated still fail to support transformation. Nature of unknown factor undetermined.

6. Properties of serum - stable; may be stored in refrigerator many months and retain original effectiveness.

Recognition of serum factors and properties facilitated standardization of cultural conditions required for consistent and reproducible results.

C. R strain (R36A) - derived from Pneumococcus Type II

1. Characteristics - relatively fixed in R phase; never spontaneously reverts. Repeated attempts to cause reversion unsuccessful. Strain susceptible to transformation to variety of different S types. (I, III, VI, and XIV)

2. Dissociation - on serial transfer in blood broth R strain undergoes spontaneous dissociation → number of varients distinguishable by colony form. Only R36A susceptible; other variants all inactive. Differences in responsiveness of different R variants to same stimulus. Emphasizes care requisite in selection of suitable R-variant.

3. Intracellular enzyme - pneumococcal cells release upon autolysis an enzyme that destroys activity of transforming extracts. Important in the cultural conditions of inducing transformation and in the extraction of transforming principle from pneumococcal cells.

To obtain consistent reproducible results, bear in mind:

1. R strain may undergo spontaneous dissociation giving rise to other variants incapable of responding to transforming stimulus.

2. Pneumococcal cells contain intracellular enzyme which when released destroys activity of transforming principle.

Hence, important to select reactive strain and to prevent destructive changes associated with autolysis.

D. Transforming principle

Quantitative titration of activity of transforming material.

Sterile material serially diluted in saline at neutral pH. 0.2 cc. of each dilution added to three or more tubes containing 2 cc. of broth to which 10 per cent serous fluid has been added. Tubes seeded with 0.05 cc. of a 10^{-4} dilution of a 5-8 hour blood broth culture of the R strain (R36A).

Cultures incubated 37° for 18-24 hours. Anti-R in serum medium causes the R cells to agglutinate during growth. The agglutinated clumps settle to bottom of tube leaving clear supernatant. When transformation occurs, encapsulated S cells unaffected by the R antibodies grow diffusely, supernatant becoming uniformly turbid. By inspection alone can distinguish tentatively between positive and negative results.

D. (continued)

All cultures plated on blood agar for further identification. Differentiation of colonies of parent R cells and those of transformed S organisms striking - latter large, glistening mucoid typical Type III.

Slide B Colonies

Slide 2 - Colonies

III. Preparation of transforming principle

A. Source material

1. 75 liter lots of culture of Type III pneumococcus. Young, actively growing (16 hr.)

2. Collected on Sharples centrifuge.

3. Cells resuspended in saline - heat-killed 65°C. 30' This temperature inactivates enzyme known to destroy t. p.

B. Extraction

1. Heated cells washed 3 times with saline, removing large excess capsular polysaccharide, much protein, ribonucleic acid and C polysaccharide - 10-15 per cent loss of transforming material.

2. Extracted with saline containing 0.5 per cent sodium desoxycholate, by mechanical shaking. Repeated 3 times.

3. Extracts combined, precipitated by excess ethyl alcohol. Ppt - floating fibrous mass. Redissolved in saline.

C. Deproteinization and Removal of S polysaccharide

1. Preliminary deproteinization by Sevag chloroform method. About 3 times

2. SIII Enzyme hydrolyzes Type III capsular polysaccharide. Enzymatic breakdown usually complete 4-6 hours - evidenced by loss of serological activity.

3. Reprecipitated by alcohol. Deproteinization repeated until no further protein-chloroform get at interface.

D. Alcohol fractionation

1. Dropwise addition of absolute ethyl alcohol with constant stirring

2. At critical concentration - 0.8 - 1.0 volume - fibrous strands separate out and collect on rod.

3. Repeated 4-5 times

4. Yield of fibrous material = 10-25 mgm. per 75 liter lot and represents major portion of active material in crude extract.

E. Effect of temperature

1. Extraction less efficient but activity best preserved when procedures are carried out at 0°-4°C.

IV. Analysis of Purified Material

A. General properties

1. Saline solutions (1 mg./cc) - colorless, viscous, clear in diffuse light. In strong transmitted light, silky sheen on stirring.

2. Preservation - saline solutions retain activity 2-4°C. at least 3 months. Longer periods in frozen state in CO_2 cabinet @ -10°. In aqueous solution rapid decrease in activity; completely inert in few days.

Material precipitated from saline solution by alcohol and stored under alcohol remain active for long periods.

3. Effect of temperature - withstands 30-60 min. 65°C. Higher temperatures not tested.

4. Effect of pH - activity rapidly lost at acidities greater than pH 5. Best preserved at neutral or slightly alkaline reaction.

B. Qualitative Chemical Tests

1. Biuret and Millon tests negative - even on dried material

2. Dische diphenylamine reaction for desoxyribose strongly positive

3. Orcinol test (Bial) for ribose weakly positive. However, in similar concentrations pure preparations of desoxyribonucleic acid of animal origin prepared by different methods give Bial reaction of corresponding intensity.

4. Lipids - no specific tests. Crude material repeated extracted with alcohol and other -12°C. without loss of activity. Repeated alcohol precipitation and treatment with chloroform results in no decrease in activity.

C. Elementary Chemical Analysis (Dr. Elek)

Slide 3

N/P ratio varies from 1.58 - 1.75. Average value 1.67.

Close agreement with that calculated on the basis of the theoretical structure of sodium desoxyribonucleate.

N/P ratio indicates little protein or other substance containing N or P present, otherwise ~~ration~~ ratio would be considerably different.

D. Enzymatic Analysis

1. Crystalline enzymes (Northrop and Kunitz)

Trypsin, chymotripsin and ribonuclease - no effect on activity.
∴ not ribo-n.a or protein susceptible to action tryptic enz
Pepsin not tested because pH required for action itself inactivates.

2. Crude enzymes

Dog intestinal mucosa - Levene and Dillon - polynucleotidase preparation.
Rabbit bone phosphatase - Martland and Robison
Swine kidney phosphatase - Albers, A. and E.
Pneumococcus autolysates
Normal dog and rabbit serum.

Slide 4 Preparation of Crude Enzymes Tested for their Effect on Tp-

(Dr. Mirsky - desoxyribonucleate)

Parallelism between enzyme that causes depolymerization of known samples of desoxyribonucleic acid and that which destroys activity of transforming principle. Irrespective of phosphatase or esterase activity, only those preparations shown to contain an enzyme capable of depolymerizing authentic samples of DRNA were found to inactivate transforming principle.

3. Differential Heat Inactivation of Dog and Rabbit Serum 1st on Tp-

Slide 5

Greenstein and Jenrette shown that sera of several different mammalian species contain enzyme which causes depolymerization of D.R.N.A. Greenstein has termed this desoxyribonucleodepolymerase. ~~Action of enzyme measured by decrease of viscosity of mixtures of~~ enzyme and nucleate in viscosimeters.

Slide 5

Dog and rabbit serum, tested on partially purified preparation of transforming material.

Data show that both dog and rabbit serum unheated is capable of completely destroying transforming activity. However, when dog serum is heated at 60° or higher for 30 minutes there is no loss of transforming activity. That is, the serum enzyme responsible for destruction is completely inactivated at 60°C. In contrast exposure to 65°C. is required for complete inactivation of the corresponding enzyme in normal rabbit serum.

Same samples of serum also tested for depolymerase activity on sodium desoxyribonucleate.

5 shows differential Heat inactivation of Enzyme in Dog + Rab. Sera for Transforming Tp.
6. Showing Same Differences in Temperature effect on activity of Enzymes
~~# 2 Agents~~ that depolymerize DRNA —

Slide 6

Depolymerization followed reduction in viscosity and progressive decrease in acid precipitability of the nucleate.

Data in Slide 6 show the differential heat inactivation of the depolymerase in dog and rabbit serum. With unheated serum of both species, viscosity fell to that of water in 5-7 hours.

Dog serum heated at 60 and 65°C. - no significant reduction in viscosity after 22 hours.

On the other hand, heating rabbit serum at 60°C. merely reduced the rate of reaction. The depolymerase was completely destroyed at 65°C.

Read → Thus, striking parallelism between temperature of inactivation of depolymerase and that of the enzyme which destroys T.P. (shown in Slide 5). Difference in temperature of inactivation not a general property of all enzymes in these sera as evidenced by experiments on heat inactivation of esterase in these same samples. In the latter instance, the results are the reverse of those observed with depolymerase, since the esterase of rabbit serum is almost completely destroyed at 60°C. while that of dog serum is only slightly affected by exposure to this temperature.

4. Inhibition by NaF.

Of various substances tested only sodium fluoride found to exert significant inhibition known to destroy transforming principle. Irrespective of whether this enzyme derived from pneumococcal cells, dog intestinal mucose or normal animal sera, its activity is inhibited by fluoride. Similarly, found that fluoride inhibits the enzyme causing depolymerization of D.R.N.A.

5. The fact that transforming activity is destroyed only by enzyme preparations known to contain depolymerase for D.R.N.A. and further fact that in both instances the enzymes concerned are inactivated at the same temperature and are inhibited by fluoride provide additional evidence for the belief that transforming principle is a nucleic acid of the desoxyribose type.

E. Serological Analysis

1. Progressive loss of serological activity with chemical purification, without corresponding loss of activity.

2. Highly purified transforming principle. Only faint tract reactions with high titer Type III rabbit antiserum. Indicates elimination during purification of such serologically reactive substances as S, P, and C.

3. Contrast between biological activity and serological reaction.

F. Physical Chemical Studies (Prep. #44)

1. Ultracentrifugation (Dr. Rothen)

a. Single, sharp boundary - indicating substance homogeneous and molecules uniform in size and very asymmetric.

b. Biological activity sedimented at same rate as boundary.

c. Molecular weight - cannot be accurately determined pending measurement of diffusion constant and partial specific volume. However, Tennent and Vilbrandt have determined the diffusion constant of several preparations of T.N.A., the sedimentation rate of which closely agrees with that observed in the present study. Assuming that the asymmetry of the molecules is the same in both instances, it is estimated that the molecular weight is of the order of 500,000.

2. Electrophoresis (Dr. Shedlovsky)

a. Single electrophoretic component of relatively high mobility comparable to that expected of a nucleic acid.

b. Transforming activity associated with this fast-moving component giving boundary.

c. Thus, in both the centrifugal and electrical fields, behavior of active material consistent with the concept that biological activity is a property of the highly polymerized nucleic acid.

3. Ultraviolet spectroscopy (Dr. Lavin)

a. Absorption curves showed maxima at 2600 Å and minima in the region of 2350 Å.

b. Findings characteristic of a nucleic acid.

G. Quantitative Determination of Biological Activity

1. Various preparations of highly purified material active in amounts ranging from 0.02 - 0.003 micrograms

2. Slide 7 Titration of activity of Prep. #44 - prepared in cold; high activity; N/P ratio = 1.58

On the basis of dry weight - 0.003 micrograms is effective in transformation.

Since the reaction system containing the 0.003 micrograms has a volume 2.25 cc. this represents a final concentration of the purified substance of 1 part in 600,000,000.

Discussion

Although the observations are limited to a single example, they acquire broader significance from the work of earlier investigators who demonstrated the interconvertibility of various pneumococcal types and showed that the specificity of the changes induced is in each instance determined by the particular type of encapsulated cells used to evoke the reaction. From the point of view of the phenomenon in general, ~~therefore,~~ it is of special interest that in the example studied, highly purified and protein-free material consisting largely, if not exclusively, of desoxyribonucleic acid is capable of stimulating unencapsulated R variants of Pneumococcus Type II to produce a capsular polysaccharide identical in type specificity with that of the cells from which the inducing substance was isolated. Equally striking is the fact that the substance evoking the reaction and the capsular substance produced in response to it are chemically distinct, each belonging to a wholly different class of chemical compounds.

The inducing substance, on the basis of its chemical and physical properties, appears to be a highly polymerized and viscous form of Sodium desoxyribonucleate. On the other hand, the Type III capsular ~~substance~~ material, the synthesis of which is evoked by this transforming agent, consists chiefly of a non-nitrogenous polysaccharide constituted of glucose-glucuronic acid

units linked in glycosidic union (22). The presence of the newly formed capsule containing this serologically reactive type-specific polysaccharide confers on the transformed cells all the distinguishing characteristics of Pneumococcus Type III. Thus, it is evident that the inducing substance and the capsular substance produced in turn are chemically distinct and biologically specific in their action and that both are requisite in determining the type specificity of the cell of which they form a part.

The experimental data presented in this paper strongly suggest that nucleic acids, at least those of the desoxyribose type, possess different specificities as evidenced by the selective action of the transforming principle. Indeed, the possibility of the existence of specific differences in biological behavior of nucleic acids has previously been suggested (23, 24) but has never been experimentally demonstrated owing in part at least to the lack of suitable biological methods. The techniques used in the study of transformation appear to afford a sensitive means of testing the validity of this hypothesis, and the results thus far obtained add supporting evidence in favor of this point of view.

If it is ultimately proved beyond reasonable doubt that the transforming activity of the material described is actually an inherent

property of the nucleic acid, one must still account on a chemical basis for the biological specificity of its action. At first glance, immunological methods would appear to offer the ideal means of determining the differential specificity of this group of biologically important substances. Although the constituent units and general pattern of the nucleic acid molecule have been defined, there is as yet relatively little known of the possible effect that subtle differences in molecular configuration may exert on the biological specificity of these substances. However, since nucleic acids free or combined with histones or protamines are not known to function antigenically, one would not anticipate that such differences would be revealed by immunological techniques. Consequently, it is perhaps not surprising that highly purified and protein-free preparations of desoxyribonucleic acid, although extremely active in inducing transformation, showed only faint trace reactions in precipitin tests with potent Type III antipneumococcus rabbit sera.

From these limited observations it would be unwise to draw any conclusion concerning the immunological significance of the nucleic acids until further knowledge on this phase of the problem is available. Unless special immunochemical methods can be devised similar to those so successfully used in demonstrating the serological specificity of simple non-antigenic

substances, it appears that the techniques employed in the study of transformation are the only ones available at present for testing possible differences in the biological behavior of nucleic acids.

Attempts to induce transformation in suspensions of resting cells held under conditions inhibiting growth and multiplication have thus far proved unsuccessful, and it seems probable that transformation occurs only during active reproduction of the cells. Important in this connection is the fact that the R cells, as well as those that have undergone transformation, presumably also all other variants and types of pneumococci, contain an intracellular enzyme which is released during autolysis and in the free state is capable of rapidly and completely destroying the activity of the transforming agent. It would appear, therefore, that during the logarithmic phase of growth when cell division is most active and autolysis least apparent, the cultural conditions are optimal for the maintenance of the balance between maximal reactivity of the R cell and minimal destruction of the transforming agent through the release of autolytic ferments.

In the present state of knowledge any interpretation of the mechanism involved in transformation must of necessity be purely theoretical. The biochemical events underlying the phenomenon suggest that the transforming

principle interacts with the R cell giving rise to a coordinated series of enzymatic reactions that culminate in the synthesis of the Type III capsular antigen. The experimental findings have clearly demonstrated that the induced alterations are not random changes but are predictable, always corresponding in type specificity to that of the encapsulated cells from which the transforming substance was isolated. Once transformation has occurred, the newly acquired characteristics are thereafter transmitted in series through innumerable transfers in artificial media without any further addition of the transforming agent. Moreover, from the transformed cells themselves, a substance of identical activity can again be recovered in amounts far in excess of that originally added to induce the change. It is evident, therefore, that not only is the capsular material reproduced in successive generations but that the primary factor, the transforming principle itself, which controls the occurrence and specificity of capsular development, is also reduplicated in the daughter cells. The induced changes are not temporary modifications but are permanent alterations which persist provided the cultural conditions are favorable for the maintenance of capsule formation. The transformed cells can be readily distinguished from the parent R forms not alone by serological reactions but by the presence of a newly formed and visible capsule which is

the immunological unit of type specificity and the accessory structure essential in determining the infective capacity of the micro-organism in the animal body.

It is particularly significant in the case of pneumococci that the experimentally induced alterations are definitely correlated with the development of a new morphological structure and the consequent acquisition of new antigenic and invasive properties. Equally if not more significant is the fact that these changes are predictable, type specific, and heritable.

Various hypotheses have been advanced in explanation of the nature of the changes induced. In his original description of the phenomenon Griffith (1) suggested that the dead bacteria in the inoculum might furnish some specific protein that serves as a "pabulum" ~~and enables~~ which enables the R form to manufacture a capsular carobhydrate.

More recently the phenomenon has been interpreted from a genetic point of view (26, 27). The inducing substance has been likened to a gene, and the capsular antigen which is produced in response to it has been regarded as a gene product. In discussing the phenomenon of transformation Dobzhansky (27) has stated that "If this transformation is described as a genetic

mutation - and it is difficult to avoid so describing it - we are dealing with authentic cases of induction of specific mutations by specific treatments ..."

Another interpretation of the phenomenon has been suggested by Stanley (28) who has drawn the analogy between the activity of the transforming agent and that of a virus. On the other hand, Murphy (29) has compared the causative agents of fowl tumors with the transforming principle of Pneumococcus. He has suggested that both these groups of agents be termed "transmissible mutagens" in order to differentiate them from the virus group. Whatever may prove to be the correct interpretation, these differences in viewpoint indicate the implications of the phenomenon of transformation in relation to similar problems in the fields of genetics, virology, and cancer research.

It is, of course, possible that the biological activity of the substance described is not an inherent property of the nucleic acid but is due to minute amounts of some other substance adsorbed to it or so intimately associated with it as to escape detection. If, however, the biologically active substance isolated in highly purified form as the sodium salt of desoxyribonucleic acid actually proves to be the transforming principle,

as the available evidence strongly suggests, then nucleic acids of this type must be regarded not merely as structurally important but as functionally active in determining the biochemical activities and specific characteristics of pneumococcal cells. Assuming that the sodium desoxyribonucleate and the active principle are one and the same substance, then the transformation described represents a change that is chemically induced and specifically directed by a known chemical compound. If the results of the present study on the chemical nature of the transforming principle are confirmed, then nucleic acids must be regarded as possessing biological specificity the chemical basis of which is as yet undetermined.

The evidence presented supports the belief that a nucleic acid of the desoxyribose type is the fundamental unit of the transforming principle of Pneumococcus Type III.